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<p>(54) Title: APPLE PROMOTERS FOR EXPRESSION OF TRANSGENES IN PLANTS (57) Abstract The present invention is directed to apple fruit-associated and <i>Thi</i> 1.3:actin fusion promoters capable of promoting the expression of heterologous genes. The invention provides apple fruit-associated and <i>Thi</i> 1.3:actin fusion promoters together with heterologous nucleic acid constructs, vectors, kits, transformation methods, transgenic plant cells and transgenic plants comprising such promoters.</p>		

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APPLE PROMOTERS FOR EXPRESSION OF TRANSGENES IN PLANTS

FIELD OF THE INVENTION

The present invention relates to novel apple fruit-associated and *Thi* 1.3:actin fusion promoters, and to heterologous nucleic acid constructs, vectors, kits, and transformation methods employing such promoters. The invention further relates to transgenic plant cells and plants transformed with heterologous nucleic acid constructs comprising an apple fruit-associated or *Thi* 1.3:actin fusion promoter.

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BACKGROUND OF THE INVENTION

40 Transcriptional regulatory sequences or promoters that regulate gene expression in plants are essential elements of plant genetic engineering. Several examples of promoters useful for the expression of heterologous genes in plants are now available (Zhu, *et al.*, 1995; Ni, *et al.*, 1995).

Most promoters are from about 500-1500 bases. Promoters for expressing a heterologous gene sequence in plants can be derived from plant DNA, *e.g.*, the cauliflower heat shock protein 80 (*hsp80*, Brunke and Wilson, 1993; U.S. Pat. No. 5,612,472), or from other sources, for example, plant viruses *e.g.*, the 35S cauliflower mosaic virus promoter, or bacteria which infect plants, *e.g.*, the nopaline synthase (*nos*) promoter (Rogers, 1991), the octopine synthase (*ocs*) promoter (Leisner and Gelvin, 1988) and the mannopine synthase (*mas*) promoter from *Agrobacterium*.

Expression of heterologous genes or selected sequences of genes in transgenic plants has typically involved the use of constitutive promoters, which drive the expression of a product throughout the plant at all times and in most tissues (*e.g.*, *hsp80*), the tomato ubiquitin promoter (Picton, *et al.*, 1993), and the raspberry E4 promoter (U.S. Pat. Nos. 5,783,393; and 5,783,394).

A limited number of inducible and/or tissue specific promoters are known. Promoters that provide fruit-specific expression include the E4 and E8 promoter from tomato (Cordes, *et al.*, 1989; Bestwick, *et al.*, 1995; U.S. Pat. No. 5, 859,330). Another fruit-specific promoter is the tomato 2AII gene promoter. It has been demonstrated that nucleic acid sequences placed under the regulatory control of the 5' non-coding region of the tomato 2AII gene (Van Haaren, 1993) are preferentially transcribed in developing fruit tissue. Fruit specific regulation of the kiwifruit actinidin promoter has been reported to be conserved in transgenic petunia plants (Lin, *et al.*, 1993).

Differential screening has been used to identify abundant transcripts in developing and ripening fruit. In banana, for example, a cDNA clone encoding a putative thaumatin-like protein is identified as among the most abundant transcripts in ripening fruit, and in kiwifruit a metallothionein-like transcript is identified as very abundant in ripening fruit (Clendennen and May, 1997; Ledger and Gardner, 1994). Abundant transcripts have also been identified in the fruit of grape, cherry, and apple (Fils-Lycaon *et al.*, 1996; Lee *et al.*, 1993).

A transcript was previously identified in Golden Delicious apple (GenBank L15194; Lee, Gardner, and Lay-Yee, 1993, Plant Physiol. 103: 1017) that is abundant in fruit and shows sequence similarity to an auxin-repressed protein (ARP) of unknown function from strawberry.

Apple is a fruit which has been the subject of a great deal of study over the past several decades (Knee, 1993). Ethylene reduction is desired by packers and shippers in order to maintain apples from over-ripening and rotting. Refrigeration, high concentrations of CO₂ and low concentrations of O₂ are currently being employed to reduce the harmful effects of ethylene during storage. Such methods suffer from the disadvantages that fruits picked at preclimacteric stages (prior to full ripening) respond better to controlled atmosphere conditions than those at mature stages and many varieties of apple suffer from chilling injury and physiological disorders due to controlled atmosphere conditions, rendering them unmarketable.

Ethylene is a plant hormone influencing many aspects of plant growth and development, and is known to play a major role in the ripening process in fruits and vegetables. A large amount of ethylene is also produced following trauma caused by chemicals, temperature extremes, water stress, ultraviolet light, insect damage, disease, or mechanical wounding. In some tissues, exposure to only a small amount of ethylene may cause an avalanche of ethylene production in adjacent plants or plant tissues such as fresh produce. This

autocatalytic effect can be very pronounced and lead to loss of fruit quality during transportation and storage.

In plants, the ethylene biosynthetic pathway is an offshoot of the methionine recycling pathway wherein S-adenosylmethionine (SAM) is converted to aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase. A bacterial enzyme, S-adenosylmethionine hydrolase (SAMase), not normally present in plant tissue, hydrolyzes SAM, thereby slowing the production of the metabolic precursor of ethylene, ACC.

Stable integration and expression of SAMase in the cells of soft fruits and vegetables has resulted in reduced ethylene production. (See, e.g., Good *et al.*, 1994; Mathews *et al.*, 1995a; Mathews *et al.*, 1995b.) Aminocyclopropane-1-carboxylic acid (ACC)-oxidase and polygalacturonase (PG) promoters have also been isolated from apple and their effects on fruit-specific gene expression evaluated (Atkinson *et al.*, 1998).

A need exists for plant promoters that are selectively functional in particular plant tissues or types of plants and which are capable of providing expression of heterologous genes in the cells of such tissues and plants.

SUMMARY OF THE INVENTION

Applicants have identified novel fruit-associated apple promoters, designated in the present application as "*Thi-1*" and "MADS2", respectively.

Applicants have also constructed a fusion promoter, designated *Thi* 1.3:actin, which comprises a 1.3 kb *Thi-1* promoter sequence component and a melon actin promoter sequence component.

In one embodiment, the invention provides an isolated nucleic acid molecule comprising an apple fruit-associated "*Thi-1*" promoter, a functional portion thereof, or a sequence complementary to it which remains stably bound to the isolated nucleic acid sequence under at least moderate, and optionally, under high stringency conditions.

In exemplary aspects of this embodiment 1.3 kb and 975 bp *Thi-1* promoters are provided, as presented in Figure 1 (SEQ ID NO:1) and Figure 2 (SEQ ID NO:2), respectively.

In another embodiment, the invention provides an isolated nucleic acid molecule comprising an apple *Thi* 1.3: actin fusion promoter or a functional portion thereof, or a sequence complementary to it which remains stably bound to the isolated nucleic acid sequence under at least moderate, and optionally, under high stringency conditions.

An exemplary *Thi* 1.3:actin fusion promoter has the sequence presented in Figure 3 (SEQ ID NO:3).

In another embodiment, the invention provides an isolated nucleic acid molecule comprising an apple fruit-associated MADS2 promoter or a functional portion thereof, or a sequence complementary to it which remains stably bound to the isolated nucleic acid sequence under at least moderate, and optionally, under high stringency conditions.

An exemplary apple fruit-associated MADS2 promoter has the sequence presented in Figure 4 (SEQ ID NO:4).

The invention also provides nucleic acid constructs having a DNA coding sequence under the transcriptional control of an apple fruit-associated or *Thi* 1.3:actin fusion promoter. The

DNA coding sequence is typically heterologous to the promoter and operably linked to the promoter to enable expression of the encoded sequence in fruit cells.

In one respect, an apple fruit-associated or a *Thi* 1.3:actin fusion promoter of the present invention can be used to modulate ethylene production in transformed fruit cells and thereby alter the ripening phenotype of transgenic fruit composed of such fruit cells.

In this embodiment of the invention, the apple fruit-associated promoters and *Thi* 1.3:actin fusion promoter described herein are employed in a method for prolonging ripening and delaying senescence of fruit from a fruit-bearing plant. In this method, a transgenic plant containing a promoter of the present invention operably linked to a heterologous DNA coding sequence is grown to the fruit-bearing stage, at which point the heterologous DNA coding sequence is expressed in the fruit of the transgenic fruit-bearing plant.

In particular, the heterologous DNA sequence encodes a product capable of reducing ethylene biosynthesis when expressed in plant cells, e.g., S-adenosyl methionine hydrolase (SAMase), amino-cyclopropane-1-carboxylic acid (ACC) deaminase, ACC oxidase antisense molecule, ACC synthase antisense molecule, ACC oxidase cosuppression molecule, and ACC synthase cosuppression molecule. Fruit produced by these transgenic plants have a modified ripening phenotype. A modified ripening phenotype refers to an alteration of the rate of ripening (e.g., prolonged ripening and delayed senescence) of a transgenic fruit relative to corresponding (i.e., non-transgenic) wild-type fruit.

In another embodiment, an apple fruit-associated or *Thi* 1.3:actin fusion promoter of the invention is used to control expression of a DNA coding sequence such as a pathogenesis related gene, e.g. polygalacturonase inhibiting protein (PGIP), glucanase and chitinase.

Additional exemplary DNA coding sequences include, but are not limited to sequences which encode, thaumatin, sucrose phosphate synthase, invertase, lycopene cyclase, antimicrobial peptides, invertase, antisense polyphenol oxidase, antisense polyphenol peroxidase, and antisense pectate lyase.

The invention further includes a method for producing a transgenic plant such as a fruit-bearing plant. In this method, a chimeric gene, typically carried in an expression vector allowing for selection in plant cells, is introduced into progenitor cells of selected plant. These progenitor cells are then grown to produce a transgenic plant bearing fruit.

The methods and results described herein demonstrate tissue-associated regulation of gene expression in transgenic plants. The tissue-associated promoters of the present invention include a DNA sequence that regulates transcription of a heterologous nucleic acid coding sequence to which it is operably linked.

The present invention also includes the use of any of the above promoters in plant transformation vectors. Such vectors can be used in any plant cell transformation method, including *Agrobacterium*-based methods, electroporation, microinjection, and microprojectile bombardment. These vectors may form part of a plant transformation kit. Other components of the kit may include, but are not limited to, reagents useful for plant cell transformation.

In another embodiment, the invention includes a plant cell, plant tissue, transgenic plant, fruit cell, whole fruit, seeds or calli containing any of the above-described promoters, chimeric genes and the corresponding expressed gene products.

These and other objects and features of the invention will become more fully apparent when the following detailed description is read in conjunction with the accompanying figures and examples.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a single-stranded depiction of a modified apple fruit-associated *Thi-1* (1.3 kb) promoter sequence (SEQ ID NO:1), as provided in pAG162. A HindIII restriction site (AAGCTT) was engineered into the 5' end and an NcoI site (CCATGG) engineered at the start codon (underlined). A putative TATA-box has been identified at nucleotides 1028-1033.

Figure 2 is a single-stranded depiction of a modified apple fruit-associated *Thi-1* (975 bp) promoter sequence (SEQ ID NO:2), as provided in pAG162a. A HindIII restriction site (AAGCTT) was engineered into the 5' end and an NcoI site (CCATGG) engineered at the start codon (underlined). A putative TATA-box has been identified at nucleotides 692-697.

Figure 3 is a single-stranded depiction of the complete nucleotide sequence of a Fuji *Thi 1.3-Actin* fusion promoter (SEQ ID NO:3), as provided in pAG-752. Restriction sites used in subcloning are underlined, HindIII and BglII at the 5' end and NcoI containing the translational start codon ATG at the 3' end. The TATA-box delineating the fusion between the Fuji *Thi 1.3* promoter and the melon actin promoter is bolded and underlined.

Figure 4 is a single-stranded depiction of a modified apple fruit-associated MADS2 promoter sequence (SEQ ID NO:4), as provided in pAG168. A putative TATA-box is identified at nucleotides 734-739 and "GAGA" boxes, consisting of GA repeats are also identified in the 5'UTR region. The start codon (ATG) is underlined, and surrounded by an NcoI site (CCATGG).

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

As used herein, the term "polynucleotide" refers to a polymeric molecule having a backbone that supports bases capable of hydrogen bonding to typical polynucleotides, where the polymer backbone presents the bases in a manner to permit such hydrogen bonding in a sequence specific fashion between the polymeric molecule and a typical polynucleotide (e.g., single-stranded DNA). Such bases are typically inosine, adenosine, guanosine, cytosine, uracil and thymidine. Polymeric molecules include double and single stranded ribonucleic acids (RNA) and deoxyribonucleic acids (DNA), and may include polymers having backbone modifications such as methylphosphonate linkages.

A nucleic acid may be double stranded, single stranded, or contain portions of both double stranded and single stranded sequence. The depiction of a single strand also defines the sequence of the other strand and thus also includes the complement of the sequence which is depicted.

As used herein, the term "recombinant nucleic acid" refers to nucleic acid, originally formed *in vitro*, in general, by the manipulation of nucleic acid by endonucleases, in a form not normally found in nature.

As used herein, the terms "chimeric gene", "chimeric gene construct" and "chimeric nucleic acid construct" are used interchangeably and refer to recombinant nucleic acid sequences which comprise a DNA coding sequence and control sequences required for expression of the coding sequence in a plant cell.

5 As used herein, the term "transgene", refers to a non-native nucleic acid sequence, usually encoding a polypeptide, introduced into a host genome using recombinant DNA techniques.

10 As used herein, the term "regulatable promoter" refers to any promoter whose activity is affected by specific environmental or developmental conditions (e.g., a tomato E4 or E8 promoter).

As used herein, the term "constitutive promoter" refers to any promoter that directs RNA production in many or all tissues of a plant transformant at most times.

15 As used herein, the term "tissue-associated promoter" refers to any promoter which directs RNA synthesis at higher levels in particular types of cells and tissues, e.g., an apple fruit-associated promoter directs RNA synthesis at higher levels in apple fruit, relative to expression levels in apple leaves.

20 As used herein, the terms "promoter" or "promoter segment" refer to a sequence of DNA that functions in a promoter disclosed herein to direct transcription of a downstream gene. The promoter will generally be appropriate to the host cell in which the target gene is being expressed. The promoter together with other transcriptional and translational regulatory nucleic acid sequences (also termed "control sequences") are necessary to express a given gene. In general, the transcriptional and translational regulatory sequences include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences.

25 By "plant promoter" is meant a promoter or promoter region (as defined above), which in its native form, is derived from plant genomic DNA. The apple fruit-associated and apple *Thi* 1.3:actin fusion promoters of the present invention are plant promoters.

30 As used herein, "promoter strength" refers to the level of promoter-regulated expression of a chimeric or heterologous gene in a plant tissue or tissues, relative to a suitable standard (e.g., a fruit-associated promoter from a particular plant, such as apple, versus a control or standard gene promoter, for example, the 35S CaMV promoter or the CsVMV promoter (Cassava Vein Mosaic Virus promoter, Verdaguer *et al.*, 1998). Expression levels can be measured by linking the promoter to a suitable reporter gene such as GUS (β -glucuronidase). Expression of the reporter gene can be easily measured by fluorometric, spectrophotometric or histochemical assays

35 (Jefferson, *et al.*, 1987a; Jefferson, 1987b; Jefferson, RA, 1989).

40 A nucleic acid sequence is "heterologous" with respect to a control sequence (*i.e.* promoter or enhancer) when it does not function in nature to regulate the same gene the expression of which it is currently regulating. Generally, heterologous nucleic acid constructs are introduced into the cell or part of the genome in which they are present, and have been added to the cell, by transfection, microinjection, electroporation, or the like. The sequences may contain a control sequence/DNA coding sequence combination that is the same as, or different from a control sequence/DNA coding sequence combination found in the native plant.

As used herein, the term "operably linked" relative to a recombinant DNA construct or vector means nucleotide components of the recombinant DNA construct or vector are in a functional relationship with another nucleic acid sequence. For example, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous.

As used herein, the term "gene" means the segment of DNA involved in producing a polypeptide chain, which may or may not include regions preceding and following the coding region, *e.g.* 5' untranslated (5' UTR) or "leader" sequences and 3' UTR or "trailer" sequences, as well as intervening sequences (introns) between individual coding segments (exons).

The term "gene", may be used interchangeably herein with the term "nucleic acid coding sequence", and the term "structural gene" which means a DNA coding region.

As used herein, the term "sequence identity" means nucleic acid or amino acid sequence identity in two or more aligned sequences, aligned using a sequence alignment program. Sequence searches are preferably carried out using the BLASTN program when evaluating the of a given nucleic acid sequence relative to nucleic acid sequences in the GenBank DNA Sequences and other public databases. The BLASTX program is preferred for searching nucleic acid sequences that have been translated in all reading frames against amino acid sequences in the GenBank Protein Sequences and other public databases. Both BLASTN and BLASTX are run using default parameters of an open gap penalty of 11.0, and an extended gap penalty of 1.0, and utilize the BLOSUM-62 matrix. [See, Altschul, *et al.*, 1997.]

The term "% homology" is used interchangeably herein with the term "% identity" herein and refers to the level of identity between two sequences, *i.e.* 70% homology means the same thing as 70% sequence identity as determined by a defined algorithm, and accordingly a homologue of a given sequence has at least about 70%, preferably about 80%, more preferably about 85%, even more preferably about 90% sequence identity over a length of the given sequence.

A preferred alignment of selected sequences in order to determine "% identity" between two or more sequences, is performed using the CLUSTAL-W program in MacVector version 6.5, operated with default parameters, including an open gap penalty of 10.0, an extended gap penalty of 0.1, and a BLOSUM 30 similarity matrix.

A nucleic acid sequence is considered to be "selectively hybridizable" to a reference nucleic acid sequence if the two sequences specifically hybridize to one another under moderate stringency hybridization and wash conditions. Exemplary conditions include hybridization conducted as described in the Bio-Rad Labs ZetaProbe manual (Bio-Rad Labs, Hercules, CA), expressly incorporated by reference herein. For example, hybridization is conducted in 1 mM EDTA, 0.25 M Na₂HPO₄ and 7% SDS at 60° C, followed by washing in 1 mM EDTA, 40 mM NaPO₄, 5% SDS, and 1 mM EDTA, 40 mM NaPO₄, 1% SDS. Hybridization conditions are further recited in Ausubel FM *et al.*, 1993, expressly incorporated by reference herein.

As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process includes both transcription and translation.

As used herein,, the terms "transformed", "stably transformed" or "transgenic" refer to a plant cell that has a non-native (heterologous) nucleic acid sequence integrated into its genome which is maintained through two or more generations.

As used herein, the term "modulate" refers to a change in biological activity. Modulation may relate to an increase or a decrease in biological activity, binding characteristics, or any other biological, functional, or immunological property of the molecule.

As used herein, the term "ethylene regulated", refers to regulation which is induced by changes in ethylene concentration in the plant. For example, promoter activity which occurs or primarily occurs, during later stages of fruit development and/or early stages of fruit ripening, is said to be ethylene regulated.

As used herein, a "plant cell" refers to any cell derived from a plant, including undifferentiated tissue (e.g., callus) as well as plant seeds, pollen, progagules and embryos.

II. Apple Promoter Isolation

Differential screening has been used to identify abundant transcripts in developing and ripening fruit. In banana, for example, a cDNA clone encoding a putative thaumatin-like protein was identified as among the most abundant transcripts in ripening fruit, and in kiwifruit a metallothionein-like transcript was identified as very abundant in ripening fruit (Clendennen and May, 1997; Ledger and Gardner, 1995). Abundant transcripts have also been identified in the fruit of grape, cherry, and apple (Tattersall *et al.*, 1997; Fils-Lycaon *et al.*, 1996; Lee *et al.*, 1993).

Random amplification of products ("RAP") screening is a PCR-based technique that allows the rapid isolation of abundant transcripts from a PCR-accessible cDNA library constructed from an mRNA of interest.

A. Construction Of PCR-Accessible cDNA Libraries

PCR accessible cDNA libraries were made using Clontech's Marathon cDNA Amplification Kit [Clontech Laboratories, Inc.: Palo Alto, California 94303-4230], following the manufacturer's protocol. Briefly, after first and second-strand cDNA synthesis, adaptors were ligated to the polished ends of the double-stranded cDNA. This cDNA library served as a PCR-accessible cDNA library for rapid amplification of cDNA 5' or 3' ends.

After the libraries were constructed, modified adaptors were ligated to the digested DNA fragments, such that the adaptor thereby defines one end of the PCR template. A pair of nested gene specific primers and a pair of nested adaptor primers were used to amplify a specific product or products. A chemical modification of the adaptor, as well as the use of touchdown PCR parameters, minimized amplification of non-specific products.

Touchdown PCR parameters are thermal cycling parameters in which the annealing temperature is initially very high, and gradually decreases during subsequent cycles. Reagents and protocols for touchdown PCR are commercially available.

Upstream sequences associated with differentially expressed apple mRNAs were isolated in a series of steps. Oligonucleotide primers were designed based on the differential display fragments selectively expressed in a particular fruit tissue or fruit tissue at a particular stage of development (*i.e.*, complementary to the 5' end of the fragment), and used to walk upstream in a PCR-accessible apple genomic library.

B. RAP Screening

A PCR-accessible cDNA library was constructed using Clontech's Marathon cDNA Amplification Kit, following the manufacturer's protocol, as set forth above [Clontech Laboratories, Inc., Palo Alto, California 94303-4230]. Amplification products representing high-abundance transcripts in the mRNA pool were identified by hybridization with a labeled total cDNA probe. mRNA was isolated from particular tissues, reverse transcribed into double-stranded cDNA, blunt-ended and adaptors ligated to the double-stranded cDNA. The cDNA library was serially diluted 10-fold, at least ten times, in order to reduce non-abundant transcripts to undetectable levels. Each serial dilution was used as a template in replicated PCR reactions. Products were amplified using an oligonucleotide complimentary to the adaptor sequence and a cDNA synthesis primer for random amplification. The products of the PCR reactions were separated by agarose gel electrophoresis and analyzed by Southern blot, and visualized on an ethidium bromide-stained gel. The blots were probed with radiolabeled first-strand cDNA, from the same mRNA used to construct the library. Amplification fragments which hybridize strongly to the labeled cDNA probe represent abundant transcripts in the tissue that served as the source for RNA extraction. These fragments were cloned and their expression pattern further analyzed by Northern hybridization.

III. Apple Fruit-Associated *Thi-1* Promoters

cDNA libraries were generated using RNA isolated from post-harvest Fuji apple fruit. The libraries were made using Clontech's Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA), and served as PCR-accessible libraries for random amplification of products (RAP) and screening by hybridization (post harvest fruit library).

Using the RAP technique, a cDNA fragment was identified as being abundantly expressed in Fuji apple fruit (post harvest). The Fuji apple cDNA fragment (LIB7) was cloned, sequenced and found to exhibit nucleotide sequence homology to plant thiamine biosynthetic enzymes (*Thi*).

Thiamine or vitamin B-1, is an essential constituent of all cells since it is a cofactor for two enzyme complexes involved in the citric acid cycle, pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase. Plant thiamine biosynthetic enzymes (*Thi*) are known to exist in higher plants, however, the biosynthetic pathway for thiamine in plants has not been well characterized.

A *Thi-1* gene homologous to yeast and plant genes encoding an enzyme belonging to the pathway of thiamine biosynthesis has been cloned from maturing citrus fruit, and the expression of the gene shown to gradually increase in the peel during natural fruit maturation and in response to ethylene (Jacob-Wilk, 1997; GenBank Accession Number Z82983).

Two cDNAs representing members of the maize *Thi1* gene family shown to be located in a plastid membrane fraction, have also been cloned and characterized (Belanger, FC *et al.*, 1995).

A cDNA clone, pAgthi1, encoding a gene product involved in thiazole biosynthesis, was isolated from a library made from poly(A) RNA from actinorhizal nodules of *Alnus glutinosa* by differential screening with nodule and root cDNA, respectively. The corresponding gene, agthi1, was shown to be expressed at high levels in nodules and shoot tips of *A. glutinosa*, while having low expression levels in roots, flowers, and developing fruits (Ribeiro A, *et al.*, 1996; GenBank Accession Number X97434).

The Fuji apple transcript was found to be very abundant in fruit, detectable in leaf tissue, and almost undetectable in root and ovary by Northern blot analysis. Use of the fragment as a probe on Fuji and Gala genomic Southern blots indicated two similar copies of *Thi* in both the Fuji and Gala apple genomes (See Example 1).

The *Thi* transcript present in Fuji apple fruit was determined to be encoded by *Thi-1*, and *Thi-1* sequence-specific primers were designed that preferentially amplify upstream sequences associated with the *Thi-1* gene, and used to walk upstream in a PCR-accessible Fuji apple genomic library. 1.2 and 1.0 kb products were amplified from PCR-accessible Fuji genomic libraries, assembled as a contiguous sequence, and the assembled sequence used to design primers for amplification of the complete *Thi-1* upstream promoter sequence directly from Fuji genomic DNA.

1.3kb (SEQ ID NO:1, Figure 1) and 975bp (SEQ ID NO:2, Figure 2) *Thi-1* promoters were isolated and characterized. The nucleotide sequence of the 1.3kb fragment was found to have nucleotide sequence homology to 5S ribosomal RNA genes, while the same 5S region was removed from the 5' end of the 975bp fragment. The 1.3 kb *Thi-1* promoter sequence was also fused to a melon actin promoter at the TATA-box, resulting in a hybrid fusion promoter construct designated, "*Thi* 1.3:actin".

The amplified *Thi-1* promoters were digested to produce the appropriate cohesive ends and cloned into compatible sites in a reporter gene construct, comprised of the promoter translationally fused with GUS and containing the nos3' terminator. The resulting constructs were designated pAG162, pAG162a and pAG752, respectively.

A basic BLASTN search (<http://www.ncbi.nlm.nih.gov/BLAST/>) of non-redundant nucleic acid sequence databases through NCBI (<http://www.ncbi.nlm.nih.gov/index.html>) revealed no significant matches to the 1.3 kb and 975bp *Thi-1* promoters, as presented in (SEQ ID NO:1, Figure 1) and (SEQ ID NO:2, Figure 2), respectively.

A. Fusion Promoter Comprising a 1.3 kb Apple *Thi-1* Sequence And A Melon Actin Sequence.

The Fuji *Thi* 1.3 promoter was fused to a melon actin promoter at the TATA-box, as detailed in Example 2. The complete sequence of the Fuji *Thi* 1.3 actin fusion (SEQ ID NO:3), as it exists in pAG752 is presented in Fig.3. *Hind*III and *Nco*I restriction sites were engineered into the 5' and 3' ends of the promoter fragment, respectively, to aid in subcloning.

IV. Apple Fruit-Associated MADS2 Promoter

cDNA libraries were generated using RNA isolated from developing apple ovaries. The library was made using Clontech's Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA), following the manufacturer's protocol.

These cDNA libraries were used for rapid amplification of 5' and 3' ends (RACE; developing fruit library).

A 5' RACE reaction was performed with adaptor-specific and a MADS2 gene-specific oligonucleotide primers, and a 5' RACE product of 400bp isolated, cloned, and sequenced. The 5' RACE product showed homology to a known 5'UTR, MADSBOX and variable region of the MADS2 cDNA clone.

MADS2 has been identified as an ovary-specific transcript in Fuji apple (Sung and An, 1997). MADS2 is a homeobox gene, and the gene product is predicted to be involved in early fruit development. Upstream regulatory sequences associated with the MADS2 gene were isolated for use as a promoter to drive the expression of heterologous genes in the developing fruit of apple and other tree fruit. Seven MADS-box genes have been identified from apple and shown to be expressed in different parts of the fruit (Yao, J et al, 1999).

A putative translational start site was identified and MADS2-specific oligonucleotides designed to walk upstream in a PCR-accessible Fuji apple genomic library (using GenomeWalker, Clontech), resulting in isolation of 750 bp product. The 750 bp sequence was used to design primers for a second upstream walk which led to isolation of a 720 bp product, as detailed in Example 2.

The 400 bp RACE product, and the 750 bp and 720 bp GenomeWalker products were assembled as a contiguous sequence which was used to design primers to amplify the complete MADS2 upstream sequence directly from genomic DNA. A complete MADS2 promoter amplified from Fuji apple genomic DNA is presented in Figure 4 (SEQ ID NO:4).

In order to confirm promoter activity, the MADS2 promoter was subcloned into a reporter gene construct in translational fusion with GUS and containing the nos terminator sequence and the resulting MADS2 promoter::reporter gene construct was designated pAG168.

The MADS2 promoter sequence exhibits a high A/T percentage, contains a putative TATA box and two "GAGA" boxes that are known to exist in the 5'UTR of the Fuji MADS2 cDNA. The nucleotide sequence of the MADS2 promoter, as it exists in pAG168, is presented in Figure 4 (SEQ ID NO:4). A Basic BLASTN search (<http://www.ncbi.nlm.nih.gov/BLAST>) of non-redundant nucleic acid sequence databases through NCBI (<http://www.ncbi.nlm.nih.gov/index.html>) with the MADS2 promoter sequence revealed sequence identity between the 5'UTR of the MADS2 cDNA (*Malus domestica* sequence, GenBank accession number U78948; Sung, Yu and An, unpublished), and nucleotides 865 to 971 of the 1038 bp MADS2 promoter sequence (SEQ ID NO:4).

V. Vectors For Transforming Plant Cells

The present invention further provides vectors suitable for the transformation of plants. The vectors, chimeric genes and DNA constructs of the present invention are also useful for the expression of heterologous genes. Transgenic plants, transgenic plant cells and transgenic fruit,

carrying the chimeric genes of the present invention, may be a useful source of recombinantly-expressed material.

The apple fruit-associated and *Thi-1* 1.3:actin fusion promoters of the invention find utility in chimeric gene constructs for the fruit-associated expression of heterologous structural genes operably linked to the promoters. The methods and results described herein are directed to gene expression under the control of an apple fruit-associated or apple *Thi-1* 1.3:melon actin fusion promoter of the invention, in transgenic plant cells. The apple fruit-associated and *Thi-1* 1.3:actin fusion promoters of the invention include a region of DNA that promotes transcription of a gene operably linked thereto, in transformed plant cells.

Using known, routine DNA manipulation techniques such as those described in Sambrook *et al.* (1989), heterologous nucleic acid constructs can be made whereby a foreign structural DNA sequence of interest, or gene, is placed under the regulatory control of an apple fruit-associated or apple *Thi-1* 1.3:melon actin fusion promoter of the invention.

Techniques for the construction of expression vectors or heterologous nucleic acid constructs suitable for transformation into plants are known to those of ordinary skill in the art. (See, for example, Houck and Pear, 1990, and Becker, *et al.*, 1992).

For expression in plants, the expression vectors of the invention may be constructed to contain an insertion site for a DNA coding sequence of interest. The transcription of such inserted DNA is then under the control of an apple fruit-associated or apple *Thi-1* 1.3:melon actin fusion promoter of the invention.

Such expression vectors may have single or multiple transcription termination signals at the 3' end of the DNA sequence being expressed. The expression cassette may also include, for example, (i) a DNA sequence encoding a leader peptide (*e.g.*, to allow secretion or vacuolar targeting), (ii) translation termination signals, (iii) selectable marker genes for use in plant cells, (iv) sequences that allow for selection and propagation in a secondary host, such as an origin of replication and a selectable marker sequence.

Selectable marker genes encode a polypeptide that permits selection of transformed plant cells containing the gene by rendering the cells resistant to an amount of an antibiotic that would be toxic to non-transformed plant cells. Exemplary selectable marker genes include the neomycin phosphotransferase (*npII*) resistance gene, hygromycin phosphotransferase (*hpt*), bromoxynil-specific nitrilase (*bxn*), phosphinothricin acetyltransferase enzyme (BAR) and the spectinomycin resistance gene (*spt*), wherein the selective agent is kanamycin, hygromycin, geneticin, the herbicide glufosinate-ammonium ("Basta") or spectinomycin, respectively.

Typical secondary hosts include bacteria and yeast. In one embodiment, the secondary host is *Escherichia coli*, the origin of replication is a *colEI*-type, and the selectable marker is a gene encoding ampicillin resistance. Origin of replication and selectable marker sequences operative in secondary hosts are well known in the art and many are commercially available (*e.g.*, Clontech, Palo Alto, CA; Stratagene, La Jolla, CA).

The vectors of the present invention are useful for fruit tissue-associated expression of nucleic acid coding sequences in plant cells. For example, a selected peptide or polypeptide coding sequence can be inserted in an expression vector of the present invention which has, *e.g.*, a *Thy-1*, MADS2 or *Thi* 1.3:actin fusion promoter sequence. The vector is then transformed into

host cells, and the host cells cultured under conditions to allow the expression of the protein coding sequence. In some cases, the expressed peptide or polypeptide is isolated from the cells. Transformed plant progenitor cells can also be used to produce transgenic plants bearing fruit.

Further, the invention includes a method for producing a transgenic fruit-bearing plant, where fruit produced by the plant has a modified phenotype. In this method a heterologous gene construct is introduced (e.g., by transformation) into progenitor cells of the plant. An exemplary heterologous gene construct is composed of (i) a DNA sequence encoding a gene product effective to modify a phenotypic characteristic of the plant, e.g., to reduce ethylene biosynthesis in fruit produced by the plant, operably linked to (ii) an apple promoter of the invention whose expression is fruit-associated. The DNA sequence is heterologous to the promoter and the chimeric gene contains the appropriate regulatory elements necessary for expression in a plant cell. Transformed progenitor are grown to produce a transgenic plant bearing fruit. The method further includes transforming progenitor cells of the plant with a vector containing a selectable marker and the heterologous gene.

It will be understood that the vectors described herein may form part of a plant transformation kit. Other components of the kit may include, but are not limited to, reagents useful for plant cell transformation.

VI. Methods Of Transforming Plant Cells

Chimeric genes containing an apple fruit-associated or *Thi* 1.3: actin fusion promoter of the invention, e.g., a *Thi-1*, MADS2 or *Thi* 1.3:action fusion promoter may be transferred to plant cells by any of a number of plant transformation methodologies, including *Agrobacterium*-based methods [Ranier *et al.*, 1990 (rice); McCormick *et al.*, 1986 (tomato); Norelli *et al.*, 1996 (apple)], electroporation, microinjection, and microprojectile bombardment. (See, e.g., Comai and Coning, 1993; Klein, *et al.*, 1988; Miki, *et al.* 1987; Bellini, *et al.*, 1989).

In one embodiment, chimeric genes are introduced into plants by way of a T-DNA-less Ti plasmid carried by *Agrobacterium tumefaciens*, followed by co-cultivation of the *A. tumefaciens* cells with plant cells. In such cases, vectors for use in the invention contain a selectable marker gene, T-DNA border regions from *Agrobacterium tumefaciens*, a heterologous gene of interest, and other elements as desired. Exemplary *Agrobacterium* transformation vectors are commercially available from Clontech (Palo Alto, CA) and further described by An, *et al.*, 1985.

Other suitable vectors may be constructed using the promoters of the present invention and standard plant transformation vectors, which are available both commercially (Clontech, Palo Alto, CA) and from academic sources [Salk Institute, Plant Biology Labs; Texas A & M University; Waksman Institute, Rutgers, The State University of New Jersey, Piscataway, NJ].

Another embodiment is based on microprojectile bombardment using microparticles loaded with DNA which are bombarded into the cells using "gene gun" technology. (See, e.g., Robinson, HL and Torres, CA, 1997).

When electroporation or microprojectile bombardment transformation techniques are utilized, the transformation vector generally contains the heterologous gene of interest and a selectable marker gene construct to determine whether the transformation event was successful.

Transformed plant cells are obtained as a result of the transformation of the plant cells with a heterologous gene construct comprising an apple fruit-associated or *Thi* 1.3:actin fusion promoter of the invention operably linked to a heterologous gene. The plant cells are cultured in medium containing the appropriate selection agent to identify and select for plant cells which express the chimeric gene. After plant cells that express the chimeric gene are selected, whole plants are regenerated from the transgenic plant cells. Techniques for regenerating whole plants from transformed plant cells are known in the art.

The invention further includes a method for producing a transgenic plant such as a fruit-bearing plant. In this method, a chimeric gene, typically carried in an expression vector allowing selection in plant cells, is introduced into progenitor cells of a plant. These progenitor cells are then grown to produce a transgenic plant bearing fruit.

Preferred plants suitable for transformation using an apple fruit-associated or *Thi* 1.3:actin fusion promoter of the invention include, but are not limited to, apple, tomato, pineapple, grape, raspberry, strawberry, kiwi fruit, avocado, melon, mango, papaya, apple, peach, pear, cherry, citrus, date palm, plantain, soybean, cotton, alfalfa, oilseed rape, flax, sugar beet, sunflower, potato, tobacco, maize, wheat, rice, nuts and lettuce.

In one exemplary embodiment, cotyledon explants of a commercial cantaloupe variety (*Cucumis Melo*, Muskmelon) are transformed according to known methods (Fang and Grumet, 1990; Valles and Lasa, 1994; Dong, *et al.*, 1991; Gonsalves, *et al.*, 1994; Yoshioka, *et al.*, 1992; Ayub, *et al.*, 1996), using the a disarmed *Agrobacterium* strain to introduce the above-described binary vectors into plants. The disarmed *Agrobacterium* strain is co-cultivated with melon cotyledon tissue explants, and primary transformants selected on the basis of their capacity to regenerate and develop roots on media containing the antibiotic, kanamycin.

In other exemplary embodiments, *Agrobacterium* transformation methods as described for apple, rice, tomato, apple are used to transform plant cells using an apple fruit-associated or *Thi* 1.3:actin fusion promoter of the invention. [See, *e.g.*, Sagi *et al.*, 1995 (banana); Ranier *et al.*, 1990 (rice); McCormick *et al.*, 1986 (tomato); and Norelli *et al.*, 1996 (apple).]

VII. Heterologous Genes

Any structural gene of interest may be placed under the regulatory control of an apple fruit-associated or *Thi* 1.3:actin fusion promoter of the invention. The structural gene may encode a polypeptide of interest or other gene product.

According to the methods of the present invention, heterologous genes may be operably linked to such an apple fruit-associated or *Thi* 1.3:actin fusion promoter.

In one aspect, the apple fruit-associated promoters of the invention are used to modulate ethylene production in transformed cells, and thereby alter the ripening and delay senescence of transgenic fruit composed of such cells.

In this embodiment of the invention, the promoters described herein are employed in a method for prolonging ripening and delaying senescence of fruit from a fruit-bearing plant, *e.g.*, apple. In this aspect of the invention, transgenic plant cells containing the promoters of the present invention are grown to produce a transgenic plant bearing fruit.

In particular, plant cells are transformed with a heterologous nucleic acid construct encoding a product capable of reducing ethylene biosynthesis when expressed in plant cells (*e.g.*, S-adenosyl-methionine hydrolase (SAMase, Ferro *et al.*, 1995; Hughes *et al.*, 1987), aminocyclopropane-1-carboxylic acid (ACC) deaminase, ACC oxidase antisense molecule, ACC synthase antisense molecule, ACC oxidase cosuppression molecule, ACC synthase cosuppression molecule), which is under the control of an apple promoter of the invention. Fruit produced by these transgenic plants has a modified ripening phenotype, as described in co-owned U.S. Pat. Nos. 5,859,330; 5,783,394; 5,783,393; 5,723,746; 5,589,623; 5,416,250 and 5,750,864, expressly incorporated by reference herein.

A modified ripening phenotype refers to an alteration in the rate of ripening; characterized by an increased ripening time course, or prolonged ripening and the delayed senescence of, a transgenic fruit relative to corresponding (*i.e.*, non-transgenic) wild-type fruit.

In another aspect, the apple fruit-associated promoters of the invention are used to increase the resistance of a plant to pathogens. In such cases, the nucleic acid coding sequence can correspond to a pathogenesis related gene, such as polygalacturonase inhibiting protein (PGIP), glucanase or chitinase.

In a further aspect, nucleic acid coding sequences which are placed under the control of an apple fruit-associated or *Thi* 1.3:actin fusion promoter of the invention include sequences which affect: (i) flavor (*e.g.*, thaumatin; GenBank); (ii) pigmentation (*e.g.*, products that modify lycopene synthesis, such as lycopene cyclase; GenBank); (iii) enzymes or other catalytic products (such as, ribozymes or catalytic antibodies) that modify plant cell processes; (iv) enzymes that inhibit degradation of ripened fruit (*e.g.*, antisense polyphenol oxidase and antisense polyphenol peroxidase (to inhibit browning) and antisense pectate lyase (to inhibit softening); (vi) antimicrobial peptides, (vii) sucrose accumulating genes, such as the sucrose phosphate synthase gene (GENBANK) and (viii) genes which affect the metabolism of sucrose (*e.g.*, invertase).

VIII. Identification And Evaluation Of Transformants

Following transformation, transgenic plant cells are assayed for expression of a transgene which is operably linked to an apple fruit-associated or *Thi* 1.3:actin fusion promoter of the invention. Transgenic plant cells may be initially selected by their ability to grow in the presence of a selective agent, such as the aminoglycoside antibiotic, kanamycin.

Expression of a transgene may also be determined by analysis of DNA, mRNA, and protein, associated with the expression of the transgene. The assays are typically conducted using various plant tissue sources, *e.g.*, leaves, stem, ovaries or fruit.

A. Construction Of Plant Transformation Vectors And Evaluation Of Promoter Activity Using Reporter Constructs

The relative activity of apple fruit-associated and *Thi* 1.3:actin fusion promoters of the invention was evaluated in a transient assay system using a reporter gene, exemplified by GUS (β -glucuronidase), effective to evaluate the tissue-associated regulatable expression from the promoters. Expression of GUS protein is easily measured by fluorometric, spectrophotometric or histochemical assays (Jefferson, 1987a).

Recombinant nucleic acid constructs comprising; pAG162, pAG162a, pAG752 and pAG168 were prepared using the isolated promoter sequences and techniques routinely employed by those in the art, then introduced into apple plant cells by particle bombardment, as detailed below in Example 3.

5 The promoter activity of various GUS constructs is then evaluated in transient assays for GUS expression. Gold particle suspensions of each construct are prepared and used to bombard sterilized apple, peach and pear fruit which is immature or at various stages of maturity including fully mature ripe fruit.

10 B. Methods Of Detecting Apple Promoter-Driven Gene Expression

Transgenic plants are assayed for their ability to synthesize mRNA, DNA, protein, and/or for their resistance to an aminoglycoside antibiotic, *e.g.*, kanamycin, which is associated with expression of the coding sequence that has been introduced into plant cells under the control of an apple fruit-associated or *Thi* 1.3:actin fusion promoter of the invention. The assays are typically conducted using various plant tissue sources, *e.g.*, leaves, stem, or fruit

15 Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA, dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein.

20 The following examples illustrate, but are in no way intended to limit the scope of the present invention.

Materials And Methods

25 DNA Plasmids And Agrobacterium Binary Vector Construction

Biological reagents were typically obtained from the following vendors: 5' to 3' Prime, Boulder, CO; New England Biolabs, Beverly, MA; Gibco/BRL, Gaithersburg, MD; Promega, Madison, WI; Clontech, Palo Alto, CA; and Operon, Alameda, CA.

30 Specific reagents employed in the particle bombardment include BioRad Biolistic PDS-1000/He system (BioRad Laboratories, Hercules, CA, USA), gold particles of 1.5 – 3.0 μ m (Aldrich, Milwaukee, WI, USA), a rupture disk: 1,100 PSI (BioRad Laboratories, Hercules, CA, USA), stop screens of 0.685 mesh (Rumsey-Loomis, Freeville, NY), macrocarriers: (Rumsey-Loomis, Freeville, NY) and X-Gluc: 5-Bromo-4-chloro-3-indoyl β -D-glucuronide cyclohexylamine salt (Rose Scientific, Edmonton, Alberta, Canada).

35 Standard recombinant DNA techniques were employed in all constructions (Adams and Yang, 1977; Ausubel, *et al.*, 1992; Hooykaas and Schilperoot 1985; Sambrook, *et al.*, 1989), expressly incorporated by reference herein.

EXAMPLE 1

40 Library Construction For Isolation Of Apple Promoters

cDNA libraries were generated using RNA isolated from developing apple ovaries and post-harvest apple fruit. Total RNA was extracted from developing and post harvest Fuji apple

fruit by the following method: Tissue was frozen and ground to a powder in liquid nitrogen in the stainless steel container of a Waring Blender. The frozen tissue powder was added to RNA extraction buffer (0.5M sodium chloride; 0.1M sodium acetate; 0.05M EDTA; 1.4%(w/v) sodium dodecyl sulfate; 2%(w/v) polyvinyl pyrrolidone 40,000; 0.2%(v/v) β -mercaptoethanol). The slurry was heated to 65°C, then homogenized with two 30s pulses of a tissue homogenizer (Polytron), and incubated for 30 min at 65°C. Cell debris was removed by straining the homogenate through cheesecloth, then further cleared by the addition of 0.2 volumes of 5M potassium acetate (pH 4.8), incubation on ice for 30 min then centrifugation at 9,000xg for 10 min at 4°C. The clear supernatant was decanted into a fresh tube, and RNA was precipitated by the addition of 0.33 volumes of 10M lithium chloride and incubation overnight at -20°C. The RNA was pelleted by centrifugation at 9,000xg for 20 min at 4°C, and the resulting RNA pellet was drained and resuspended in 0.5 ml nuclease-free water. The resuspended RNA was extracted once with phenol:chloroform (1:1), then once with chloroform: isoamyl alcohol (25:1). Finally, the RNA was precipitated by the addition of 0.1 vol. 3M sodium acetate (pH5.2) and 2.5 vol. ethanol, pelleted by centrifugation (14,000xg, 10 min), drained, and resuspended in a small volume of nuclease-free water.

Poly(A)+ RNA was isolated from 600 μ g total RNA using the Straight A's mRNA Isolation System Kit [Novagen, Inc., Madison, I]. The library itself was made using Clontech's Marathon cDNA Amplification Kit [Clontech, Palo Alto, CA] following the manufacturer's protocol. Briefly, after first and second-strand cDNA synthesis, adaptors are ligated to the polished ends of the double-stranded cDNA. This cDNA library served as a PCR-accessible library for random amplification of products (RAP) and screening by hybridization (post harvest fruit library) and also for rapid amplification of 5' and 3' ends (RACE; developing ovary library).

RAP Reactions And Hybridization

The cDNA library was serially diluted 10-fold in TE, from 10^{-1} to 10^{-10} times the original library concentration. The diluted libraries were used as a template for replicate PCR amplification reactions. Each 50 μ l reaction mix was composed of: 38.5 μ l water; 5 μ l 10X KlenTaq reaction buffer (Clontech, Palo Alto, CA); 0.5 μ l each primer (100 μ M); 0.5 μ l dNTP mix (25mM each); 5 μ l diluted library; 1 μ l KlenTaq DNA polymerase (Clontech). The primers used were MAR API (SEQ ID NO:5) and CSP (SEQ ID NO:6). The reactions were cycled in a Robocycler Gradient 96 Temperature Cycler with Hot Top Assembly (Stratagene, La Jolla, CA) using the following cycling parameters: 5 cycles [94°C, 30s; 72°C, 3min], 5 cycles [94°C, 30s; 70°C, 3min], 35 cycles [94°C, 30s; 68°C, 3min], 1 cycle [72°C, 10min], followed by a 6°C hold. A 10 μ l aliquot of each amplification reaction was separated by agarose gel electrophoresis, photographed, then transferred to nylon membrane (Nytran Plus, Schleicher and Schuell) by capillary blotting using 0.4M sodium hydroxide as the transfer fluid.

The labeled first-strand cDNA probe used in the RAP screening was synthesized from 0.5 μ g poly(A)+ mRNA in the presence of 1.5 μ M [γ - 32 P] dCTP (3000 mCi/mmol) using an oligo(dT)₁₅ primer (Promega) and 15U MMLV reverse transcriptase according to the manufacturer's instructions (Promega). The labeled first-strand cDNA was separated from

unincorporated radioisotope by G-50 column purification (ProbeQuant G-50 Micro Column, Pharmacia, New Jersey). Blots were prehybridized for 30 min in 1 mM EDTA, 0.25 M phosphate buffer (pH 7.2), 7% (w/v) SDS, and hybridized overnight at 60°C in the same solution containing the denatured probe. Hybridized filters were washed twice for 30 min each at 60°C in Wash Solution One (1 mM EDTA, 40 mM phosphate buffer, pH 7.2, 5% (w/v) SDS) and three times for 30 min each at 65°C in Wash Solution Two (1 mM EDTA, 40 mM phosphate buffer pH 7.2, 1% (w/v) SDS). The air-dried filters were subjected to autoradiography to visualize hybridizing fragments.

In one reaction (template diluted to 10^{-7}), a 600bp fragment was detected that showed a very strong signal intensity after hybridization, indicating that it represented an abundant transcript in post harvest apple fruit. The fragment was isolated, cloned, and sequenced. The abundant product exhibited nucleotide sequence homology to plant genes encoding thiamine biosynthetic enzymes (*Thi*).

The tissue distribution of Fuji apple *Thi* transcript was determined by Northern blot analysis. The *Thi* cDNA fragments were hybridized to RNA from fruit (post harvest), ovary, root, and leaf tissue on Northern blots. The 1500 nucleotide Fuji *Thi* transcript was found to be abundant in fruit, and detectable in leaf tissue, but almost undetectable in root and ovary, indicating that Fuji *Thi* is highly fruit-specific. The Fuji *Thi* cDNA fragment was also used as a probe on Fuji and Gala genomic Southern blots to determine gene copy number. The results indicate that there are two similar copies of *Thi* in both the Fuji and Gala apple genomes.

Isolation Of An Apple Fruit-Associated *Thi-1* Promoter

An oligonucleotide primer was designed based on the sequence of the *Thi*-RAP cDNA fragment, and used to walk upstream in the Fuji genome and eventually isolate an upstream regulatory region that functions as a post-harvest fruit-specific promoter. The oligonucleotide primer *Thi* 3'R (SEQ ID NO:7), was used in the amplification of upstream sequences from a PCR-accessible Fuji apple genomic library. A number of genomic DNA fragments were amplified using *Thi* 3'R (SEQ ID NO:7) and *Thi*700R (SEQ ID NO:8) primers, cloned, and sequence analysis performed. The results indicated that 2 copies of the Fuji *Thi* gene were represented in the isolated products. The two Fuji *Thi* genes differ in the size and sequence of an intron that interrupts the coding region; in *Thi-1*, the intron is 747 bp, while in *Thi-2*, 301 bp.

From the analysis of genomic fragments, a forward primer, *Thi* 4BF (SEQ ID NO:9), was designed which would amplify both *Thi* genes from the Fuji genome. Several nucleotide differences between *Thi-1* and *Thi-2* were evident in the coding region, including an approximately 4% difference at the nucleotide level, and the presence of a diagnostic restriction site (*Sma*I) in *Thi-1*, that is absent in *Thi-2*. Taking advantage of these differences in nucleotide sequence between *Thi-1* and *Thi-2*, it was determined that the *Thi* transcript present in Fuji apple fruit is encoded by *Thi-1*.

It was determined that *Thi-1* encodes a fruit-associated transcript, and *Thi-1* sequence-specific primers were designed that preferentially amplify upstream sequences associated with the fruit-associated gene. These primers were used to walk upstream in a PCR-accessible Fuji apple genomic library (Universal Genome Walker Kit, Clontech Laboratories, Inc., Palo Alto,

CA). The libraries were constructed and amplified according to the supplier's protocol. In addition to the five restriction endonucleases included in the kit, four other blunt-cutters were used to digest genomic DNA prior to adaptor ligation: BbrPI, HpaI, MscI, and SnaBI. A 1.2 kb product was amplified from the DraI library after two rounds of amplification of the Fuji genomic libraries using ThiG5'Rev1 (SEQ ID NO:10) and AP1 (SEQ ID NO:27) in the primary reaction and ThiG5'Rev2 (SEQ ID NO:11) and AP2 (SEQ ID NO:28) in the secondary reaction. Primers were designed based on the sequence of the 1.2 kb product for another upstream walk. A 1 kb product was amplified from the HpaI library after two rounds of amplification of the Fuji apple genomic libraries using ThiG5'Rev5 (SEQ ID NO:12) and AP1 (SEQ ID NO:27) in the primary reaction and ThiG5'Rev6 (SEQ ID NO:13) and AP2 (SEQ ID NO:28) in the secondary reaction. The 1.2 kb and 1 kb Genome Walker products were assembled as a contiguous sequence, and the assembled sequence used to design primers for amplification of the complete *Thi-1* upstream promoter sequence directly from Fuji genomic DNA.

A HindIII restriction site was engineered into the 5' primer, ThiG5' (SEQ ID NO:14) and an NcoI site was engineered around the start codon in the 3' primer ThiGNco3' (SEQ ID NO:15), for ease of cloning.

When ThiG5' (SEQ ID NO:14) and ThiGNco3' (SEQ ID NO:15) were used to amplify the *Thi-1* upstream regulatory region from Fuji genomic DNA, 1.3kb (SEQ ID NO:1) and 975bp (SEQ ID NO:2) amplification products were obtained. Analysis of these amplification products revealed that the 5' end of the 1.3kb fragment had nucleotide sequence homology to 5S ribosomal RNA genes, while the 975bp fragment does not. Otherwise, the two sequences were essentially identical. Both upstream sequences contain putative TATA boxes and have a high A/T percentage.

The amplified *Thi-1* promoters were digested to produce the appropriate cohesive ends and cloned into compatible sites in a reporter gene construct, comprised of the promoter translationally fused with GUS and containing the nos3' terminator, with the resulting constructs designated, pAG162 and pAG162a, respectively. The nucleotide sequence of the *Thi-1* promoters, as they exist in pAG162 and pAG162a, are presented in Figures 1 and 2, respectively.

A Basic BLASTN search (<http://www.ncbi.nlm.nih.gov/BLAST/>) of non-redundant nucleic acid sequence databases through NCBI (<http://www.ncbi.nlm.nih.gov/index.html>) reveals no significant matches to the 1.3 kb and 975bp *Thi-1* promoters, as presented in (SEQ ID NO:1, Figure 1) and (SEQ ID NO:2, Figure 2), respectively.

EXAMPLE 2

Construction of an Apple *Thi-1*:Melon Actin Fusion Promoter

The Fuji *Thi-1* 1.3 promoter was fused to a melon actin promoter at the TATA-box. Both promoters contain a canonical plant TATA-box (TATAAA), facilitating a perfect fusion between them at that site. Chimeric oligonucleotide primers were designed that were complementary to both of the promoter sequences.

A fragment containing the Fuji Thi promoter from the 5' end to the TATA box was amplified from pAG162 using 1233 (SEQ ID NO:19) and (Act)Thi_R (SEQ ID NO:17). The melon actin fragment was amplified from pAG167 using GUS5'R (SEQ ID NO:18) and (Thi)Act_F (SEQ ID NO:16). Both sets of amplifications were carried out using a PE480 thermal cycler under the following conditions: 25 cycles [94°C, 30sec.; 60°C, 30 sec.; 72°C, 90 sec.], 1 cycle [72°C, 10 min.]. The amplification of pAG167 yielded a 1.2kb fragment from the melon actin promoter containing the transcription start site, a 5'UTR intron, and the translational start site that had been engineered to contain an NcoI site for ease of subcloning.

The Fuji Thi and melon actin fragments contained a complementary overlapping region of 20 nt. The two fragments were fused by combining them in a second amplification reaction and using the end primers, 1233 (SEQ ID NO:19) and GUS5'R (SEQ ID NO:18) for amplification using a PE480 thermal cycler under the following conditions: 25 cycles [94°C, 30 sec; 60°C, 30 sec; 72°C, 150 sec], 1 cycle [72°C, 10 min]. The resulting reaction products were separated on an agarose gel, and a fragment of the correct predicted size was gel purified, digested with HindIII and NcoI, then ligated into a vector containing the reporter gene GUS and given the designation pAG752. The complete sequence of the Fuji Thi1.3 actin fusion (SEQ ID NO:3), as it exists in pAG752 is presented in Fig.3. HindIII and NcoI restriction sites were engineered into the 5' and 3' ends of the promoter fragment, respectively, to aid in subcloning.

EXAMPLE 3

Isolation Of An Apple Fruit-Associated MADS2 Promoter

mRNA was isolated from developing Fuji apple ovaries as previously described, and a PCR-accessible cDNA library was constructed (Marathon cDNA Amplification Kit, Clontech, Palo Alto, CA). A rapid amplification of cDNA 5' ends (5' RACE) reaction was performed with adaptor-specific and a MADS2 gene-specific oligonucleotide primer, apMADSPFa2, (SEQ ID NO:20). The 5' end of the Fuji MADS2 cDNA was amplified using the manufacturer's suggested conditions for RACE amplifications. A 5' RACE product of 400bp was isolated, cloned, and sequenced. The 5' RACE product showed homology to the known 5'UTR, MADSBOX and variable region of the MADS2 cDNA clone. A putative translational start site was identified and gene-specific oligonucleotides were designed to walk upstream in a Fuji apple genomic library.

MADS2-specific primers were designed from the nucleotide sequence of the 5' RACE product and used to walk upstream in a PCR-accessible Fuji apple genomic library (Universal Genome Walker Kit, Clontech, Palo Alto, CA). The libraries were constructed and screened according to the supplier's protocol. In addition to the five restriction endonucleases included in the kit, four other blunt-cutters were used to digest genomic DNA prior to adaptor ligation: BbrPI, HpaI, MscI, and SnaBI. A 750 bp product was amplified from the EcoRV library after two rounds of amplification of the Fuji apple genomic libraries using apMADSPFa3 (SEQ ID NO:21) and AP1 (SEQ ID NO:27) in the primary reaction and apMADSPFa2 (SEQ ID NO:23) and AP2 (SEQ ID NO:28) in the secondary reaction. Primers were designed from the nucleotide sequence of the 750 bp amplification product for use in another upstream walk. A

720 bp product was amplified from the HpaI library after two rounds of amplification of the Fuji apple genomic libraries using apMADSPFa4 (SEQ ID NO:22) and AP1 (SEQ ID NO:27) in the primary reaction and apMADSPFb3 (SEQ ID NO:24) and AP2 (SEQ ID NO:28) in the secondary reaction.

5 The 400 bp RACE product, and the 750 bp and 720 bp GenomeWalker products were assembled as a contiguous sequence which was used to design primers to amplify the complete MADS2 upstream sequence directly from genomic DNA. An NcoI site was engineered around the start codon, for ease of cloning in-frame with a heterologous coding sequence. A complete MADS2 promoter was amplified from Fuji apple genomic DNA with the MADS2-5' (SEQ ID
10 NO:25) and MADS2Nco (SEQ ID NO:26) primer pair.

The isolated MADS2 promoter was subcloned into a reporter gene construct which has the promoter translationally fused with GUS and contains the nos terminator sequence. The resulting MADS2 promoter::reporter gene construct was designated pAG168.

15 The MADS2 promoter sequence exhibits a high A/T percentage, contains a putative TATA box and two "GAGA" boxes that are known to exist in the 5'UTR of the Fuji MADS2 cDNA. The nucleotide sequence of the MADS2 promoter, as it exists in pAG168, is presented in Figure 4 (SEQ ID NO:4). A Basic BLASTN search (<http://www.ncbi.nlm.nih.gov/BLAST>) of non-redundant nucleic acid sequence
20 databases through NCBI (<http://www.ncbi.nlm.nih.gov/index.html>) with the MADS2 promoter sequence revealed sequence identity between the 5'UTR of the MADS2 cDNA (*Malus domestica* sequence, GenBank accession number U78948; Sung, Yu and An, unpublished), and nucleotides 865 to 971 of the MADS2 promoter, as presented in
SEQ ID NO:4.

25 EXAMPLE 4

Construction Of Plant Transformation Vectors And Evaluation Of Promoter Activity Using Reporter Constructs

30 The relative activity of the apple fruit-associated and *Thi* 1.3:actin fusion promoters was determined using a transient assay system employing the GUS reporter gene. The transient assay is based on particle bombardment of plant tissue sections with a suspension of DNA and gold particles, as described below.

35 Specific equipment and reagents employed in particle bombardment include the BioRad Biolistic PDS-1000/He system (BioRad Laboratories, Hercules, CA, USA), gold particles of 1.5 – 3.0 μ m (Aldrich, Milwaukee, WI, USA), a rupture disk: 1,100 PSI (BioRad Laboratories, Hercules, CA, USA), stop screens of 0.685 mesh (Rumsey-Loomis, Freeville, NY),
macrocarrriers: (Rumsey-Loomis, Freeville, NY) and X-Gluc: 5-Bromo-4-chloro-3-indoyl β -D-glucuronide cyclohexylamine salt (Rose Scientific, Edmonton, Alberta, Canada).

40 Solutions for use in GUS assays included: 50% Glycerol (vol/vol); 2.5M calcium chloride (CaCl_2 , 13.875 grams anhydrous CaCl_2 dissolved in 50 mls sterile dH_2O); 0.1M spermidine (0.1452 grams dissolved in 10 mls sterile dH_2O); 70% EtOH (vol/vol), 3 mls sterile dH_2O in 7 mls 200 proof ethyl alcohol; X-gluc solution (200 ml prepared by adding the components in the amounts shown in Table 1, below, to 198 ml distilled H_2O , stirring for 10

minutes or until dissolved, adjusting the pH to 7.0, dissolving 100 mg X-gluc in 2 ml DMSO, adding X-gluc/DMSO solution to the pH 7.0 solution, rinsing the X-gluc vial twice using the pH 7.0 solution, and filter sterilizing the resultant solution).

Table 1. Solutions for GUS Assay.

Component	Amount	Final Conc.
EDTA, Disodium salt	0.744 g	10.0mM
NaH ₂ PO ₄ .H ₂ O monobasic, monohydrate	1.760 g	100.0mM
K ₄ Fe(CN) ₆ .3 H ₂ O	0.042 g	0.5mM
Triton X-100	0.200 ml	0.1%

Gold particle suspensions were prepared by adding 30µl of gold particles (1.5µm to 3.0µm) to a high quality microcentrifuge tube followed by addition of 1 ml 70% EtOH. The suspension was vortexed for 20 seconds and left to stand for 25 minutes, allowing the particles to settle to the bottom of the tube so that they do not stick to the side of the tube when centrifuging, followed by centrifuging in a microcentrifuge for 6 minutes at 13,000 rpm. The supernatant was carefully removed, discarded and 500 µl sterile diH₂O added to the tube which was vortexed for 10 seconds and left standing for 25 additional minutes, followed by centrifuging in a microcentrifuge for 6 minutes at 13,000 rpm. The supernatant was again carefully removed, discarded, 500 µl sterile 50% glycerol stock added and the mixture vortexed until the particles were resuspended.

DNA solutions containing the GUS recombinant nucleic acid constructs were prepared by adding 50 µl (1 µg/µl) DNA to a microcentrifuge tube containing the gold and gently vortexing for 2-3 seconds, followed by the addition of 500 µl cold CaCl₂ (2.5M) and gentle vortexing for 2-3 seconds. This was followed by the addition of 200 µl cold spermidine (0.1M), gently vortexing at low speed (4°C), tapping the tube a couple of times every 5-10 minutes to make sure particles remained suspended for a total vortex time of about 40 minutes. Centrifuge tubes were then pulsed to a maximum of 1,500 rpm in a microcentrifuge at 4°C, three times, with the supernatant removed and discarded. 1 ml cold 70% ethanol was then added, the solution mixed and the pulse centrifuge step repeated with the supernatant removed and discarded. This pulse centrifuge step was repeated using cold 100% EtOH, followed by the addition of 350 µl cold 100% EtOH and resuspension of the particles by gentle vortexing for 2 seconds.

Fruit was prepared for particle bombardment by wiping with a towel soaked in 95% ethyl alcohol, trimming off stem and bud ends and placing in a beaker. An amount of a water/soap mix (4 drops antimicrobial soap/1000 ml H₂O) sufficient to cover the fruit was added and shaken intermittently for 15 minutes, then rinsed with diH₂O, until the soap was gone. An amount of 75% EtOH sufficient to cover the fruit was then added and shaken gently each minute for 4 minutes, the EtOH drained off and an amount of 10% bleach/2 drops Tween 20/1000 ml sufficient to cover the fruit was added and the beaker shaken intermittently for 10 minutes. The bleach was drained off and the fruit rinsed 3 times with sterile diH₂O, followed by rinsing once with sterile 500 ml diH₂O/2 ml PPM mix (Plant Preservative Mixture, Plant

Cell Technology, Washington, DC), and soaking in media consisting of filter sterilized 200 mg/l ascorbic acid and 200 mg/l citric acid, until ready to be cut. Before cutting, the fruit is blotted dry on filter paper.

Tissues for use in GUS assays include sterilized immature and mature fruit of apple, peach, and pear, at all stages of ripening from the immature ovary stage to fully mature, ripe fruit.

After cutting, the fruit was plated onto PAC1 medium containing: MS salts, B5 vitamins, glycine 2 mg/l, sucrose 3%, casein hydrolysate 100 mg/l, BA 0.5 mg/l, 2,4-D 1.5 mg/l, PPM 5 ml/l, ascorbic acid 100 mg/l, citric acid 100 mg/l, cefotaxime 200 mg/l (aa) pH 5.8, phytigel 0.25.

The fruit tissue was bombarded using GUS reporter constructs, then sealed with parafilm and left in the dark at 24°C for 22 hours. Explants were carefully transferred to clean, sterile petri plates and X-gluc solution added to completely cover the fruit. Plates were stored in an incubator at 37°C for 18 hours, the X-gluc solution drained off and 95% EtOH added to cover the fruit. Observations were made using a microscope and counting the number of GUS foci on each slice of fruit.

The relative activities of a *Thi* 1.3, *Thi* 1.0, *Thi* 1.3 :actin, MADS2 and the positive control constitutive promoter, CsVMV, were evaluated in transient assays for GUS expression in developing apple and pear fruit and in apple and pear leaves. Tables 2 through 6 present the results of transient GUS expression assays in slices of immature apple fruit (2.5 cm diameter, 5-6 seeds), developing fruit (4.5 cm diameter, 3-6 seeds) and mature fruit (5.5 cm diameter, 3-6 seeds) relative to assay results from apple leaves.

The activity of the apple promoters [(as measured by mean number of GUS foci or percent of fruit slices with foci (plus or minus the standard deviation, "SD"))] was shown to increase during fruit development, peak in mature fruit and the relative promoter activity in mature fruit was shown to be greater than the activity in leaves.

Tables 7 through 11 show the results of transient assays where GUS expression is under the control of a *Thi* 1.3, *Thi* 1.0, *Thi* 1.3 :actin, MADS2 or CsVMV promoter, respectively, in immature pear fruit (2.5 cm diameter, 9-10 seeds), developing pear fruit (3.5 cm diameter, 9-10 seeds) and mature pear fruit (5 cm diameter, 9-10 seeds).

The results were compared to the results of GUS expression assays in leaves using the same promoters. Similar to the results obtained in apple, the relative activity of the apple fruit associated promoters was greater in more mature pear fruit, at which time the activity in fruit was much greater than in the leaves. These data support the conclusion that the *Thi* 1.3, *Thi* 1.0, *Thi* 1.3 :actin and MADS2 promoters are effective to promote gene expression in mature fruit of other species, and confirm that the activity of the promoters is fruit-associated.

Table 2. Transient GUS Assay in Developing Apple Fruit and in Leaves

Promoter	Total # of fruit slices or leaves bombarded	Number of slices with GUS foci (%)	Mean # of foci/slice (SD)
Fuji Thi 1.3 (immature fruit)	30	5 (17)	2.6 (2.5)
Fuji Thi 1.3 (developing fruit)	32	2 (6)	4 (2.8)
Fuji Thi 1.3 (mature fruit)	32	11 (34)	3.3 (4.3)
Fuji Thi 1.3 (leaves)	24	4 (17)	4.3 (3.3)

Table 3. Transient GUS Assay in Developing Apple Fruit and in Leaves.

Promoter	Total # of fruit slices or leaves bombarded	Number of slices with GUS foci (%)	Mean # of foci/slice (SD)
Fuji Thi 1.0 (immature fruit)	30	1 (3)	2 (0)
Fuji Thi 1.0 (developing fruit)	32	2 (6)	4 (4.2)
Fuji Thi 1.0 (mature fruit)	32	7 (22)	1.4 (0.8)
Fuji Thi 1.0 (leaves)	24	4 (17)	3 (1.2)

Table 4. Transient GUS Assay in Developing Apple Fruit and in Leaves.

Promoter	Total # of fruit slices or leaves bombarded	Number of slices with GUS foci (%)	Mean # of foci/slice (SD)
Fuji Thi -actin (immature fruit)	30	3 (10)	5.3 (5.9)
Fuji Thi -actin (developing fruit)	32	4 (13)	1.3 (0.5)
Fuji Thi -actin (mature fruit)	32	9 (28)	1.9 (0.9)
Fuji Thi -actin (leaves)	24	4 (17)	1 (1)

Table 5. Transient GUS Assay in Developing Apple Fruit and in Leaves.

Promoter	Total # of fruit slices or leaves bombarded	Number of slices with GUS foci (%)	Mean # of foci/slice (SD)
Fuji MADS2 (immature fruit)	30	0	0
Fuji MADS2 (developing fruit)	32	1 (3)	1 (0)
Fuji MADS2 (mature fruit)	32	2 (6)	1 (0)
Fuji MADS2 (leaves)	24	2 (8)	1 (1)

Table 6. Transient GUS Assay in Developing Apple Fruit and in Leaves.

Promoter	Total # of fruit slices or leaves bombarded	Number of slices with GUS foci (%)	Mean # of foci/slice (SD)
CsVMV (immature fruit)	30	10 (33)	3 (2.8)
CsVMV (developing fruit)	32	8 (25)	2.4 (1.7)
CsVMV (mature fruit)	32	7 (22)	3.1 (3.1)
CsVMV (leaves)	24	10 (42)	2.9 (1.6)

The Fuji *Thi* 1.3, *Thi* 1.0 and *Thi* 1.3:actin promoters were particularly active in mature apple fruit. A comparison to the activity of the constitutive promoter CsVMV (Table 6) and the MADS2 promoter (Table 5) to the activity of the Fuji *Thi* 1.3 promoter (Table 2), the *Thi* 1.0 promoter (Table 3), and the *Thi* 1.3:actin promoter (Table 4) indicates that the Fuji *Thi* 1.3, *Thi* 1.0 and *Thi* 1.3:actin promoters have greater relative activity in mature apple fruit and lower relative activity in leaves.

In contrast, in developing and mature pear fruit, the *Thi* 1.3, *Thi* 1.0, and MADS2 apple promoters showed similar activity in the transient assay. All three promoters were active in developing and mature pear fruit and were significantly less active in leaves, as shown for the *Thi* 1.3 promoter (Table 7), the *Thi* 1.0 promoter (Table 8), the Fuji *Thi* 1.3:melon actin fusion promoter (Table 9), and the MADS2 promoter (Table 10).

Table 7. Transient GUS Assay in Developing Pear Fruit and in Leaves.

Promoter	Total # of fruit slices or leaves bombarded	Number of slices with GUS foci (%)	Mean # of foci/slice (SD)
Fuji <i>Thi</i> 1.3 (immature fruit)	30	0	0
Fuji <i>Thi</i> 1.3 (developing fruit)	32	8 (25)	1 (1)
Fuji <i>Thi</i> 1.3 (mature fruit)	32	12 (38)	2.2 (1.3)
Fuji <i>Thi</i> 1.3 (leaves)	27	6 (22)	1.3 (0.8)

Table 8. Transient GUS Assay in Developing Pear Fruit and in Leaves.

Promoter	Total # of fruit slices or leaves bombarded	Number of slices with GUS foci (%)	Mean # of foci/slice (SD)
Fuji <i>Thi</i> 1.0 (immature fruit)	30	1 (3)	1 (1)
Fuji <i>Thi</i> 1.0 (developing fruit)	32	11(34)	1.3 (0.6)
Fuji <i>Thi</i> 1.0 (mature fruit)	32	10 (31)	2.1 (1.4)
Fuji <i>Thi</i> 1.0 (leaves)	27	0	0

Table 9. Transient GUS Assay in Developing Pear Fruit and in Leaves.

Promoter	Total # of fruit slices or leaves bombarded	Number of slices with GUS foci (%)	Mean # of foci/slice (SD)
Fuji Thi -actin (immature fruit)	30	1 (3)	2 (2)
Fuji Thi -actin (developing fruit)	32	16 (50)	2.6 (1.6)
Fuji Thi -actin (mature fruit)	32	19 (59)	4 (3.5)
Fuji Thi -actin (leaves)	27	3 (11)	5 (2.6)

Table 10. Transient GUS Assay in Developing Pear Fruit and in Leaves.

Promoter	Total # of fruit slices or leaves bombarded	Number of slices with GUS foci (%)	Mean # of foci/slice (SD)
Fuji MADS2 (immature fruit)	30	0	0
Fuji MADS2 (developing fruit)	32	6 (19)	1.3 (0.8)
Fuji MADS2 (mature fruit)	32	13 (41)	3.7 (2.9)
Fuji MADS2 (leaves)	27	5 (19)	2 (1.4)

Table 11. Transient GUS Assay in Developing Pear Fruit and in Leaves.

Promoter	Total # of fruit slices or leaves bombarded	Number of slices with GUS foci (%)	Mean # of foci/slice (SD)
CsVMV (immature fruit)	30	6 (20)	2.3 (2.4)
CsVMV (developing fruit)	32	11 (34)	2.2 (1.5)
CsVMV (mature fruit)	32	28 (88)	3.6 (2.8)
CsVMV (leaves)	27	14 (52)	4.6 (6.9)

The results of transient assays for GUS expression in pear fruit and leaves showed that the Fuji *Thi* 1.3, *Thi* 1.0 and MADS2 promoters were particularly active in developing and mature pear fruit, with much greater activity in fruit relative to leaves. The MADS2 and CsVMV promoters were also most active in mature fruit. In addition, activity was also evident in developing fruit and leaves for MADS2 and in all tissues for CsVMV.

Although the Fuji *Thi* 1.3:melon actin fusion promoter demonstrated similar relative activity to that of the *Thi* 1.3 promoter in apple fruit (Table 4 vs. Table 2), the Fuji *Thi* 1.3:melon actin fusion promoter demonstrated greater relative activity in developing and mature pear fruit (Table 9 vs. Table 7), indicating that the *Thi* 1.3:actin fusion promoter find utility in the control of gene expression in heterologous species.

Table 12. Sequences Provided In Support Of The Invention.

Description	SEQ ID NO
Figure 1: The complete nucleotide sequence of <i>Thi-1</i> (1.3 kb) promoter. (nucleotides 1-1321)	1
Figure 2: The complete nucleotide sequence of <i>Thi-1</i> (975 bp) promoter. (nucleotides 1-985)	2
Figure 3: The complete nucleotide sequence of the Fuji <i>Thi</i> 1.3-Actin fusion as in pAG-752.	3
Figure 4: The complete nucleotide sequence of MADS2 promoter. (nucleotides 1-1038)	4
MAR API 5' CCATCCTAATACGACTCACTATA-GGGC 3'	5
CSP 5' GGGCAGGTTTCTAGAATTCAGCGGCCGC 3'.	6
<i>Thi</i> 3'R 5'-CTCGGCAACTTCCATGCCGGTGACG-3'	7
<i>Thi</i> 700R 5'-CGAGCTCGTTAAGGAAGAGATGGGC-3'	8
<i>Thi</i> 4BF 5'-TCAGGTCGCCATCATAGAGCAGTCCG-3'	9
<i>Thi</i> G5'Rev1 5'-AAGCACCCGGAAACTCTTAAACGACCAAAA-3'	10
<i>Thi</i> G5'Rev2 5'-AAATGACAGTAATTACCATGGCAGAGA-3'	11
<i>Thi</i> G5'Rev5 5'-GTCTTACTTTGAAACAGTCTCCTTAG-3'	12
<i>Thi</i> G5'Rev6 5'-ACTACGCTCCACATAAGCAAAGTCAT-3'	13
THIG5' 5'-ATTTAAGCTTTAGATCTCATGGGCGATGTGGGGATGT-3'	14
THIGNco3' 5'-GCCTTGGTTGCCATGGATTGATGCGAG-3'	15
(<i>Thi</i>)Act_F: 5' CAATTCCTTATAAACCCCGTTTC	16
(Act) <i>Thi</i> _R: 5' GGGGGTTTATAAGGGAATTGCTAAG	17
GUS5'R: GACTTCGCGCTGATACC	18
1233: AGCGGATAACAATTTACACAGGA	19
apMADSPFa2 5'-GCTGCCTCTCTGTATATGAGTATCTTTC-3'	20
apMADSPFa3 5'-ATACGGTTGTTCTGCTGAACTGATCTCT-3'	21
apMADSPFa4 5'-TTCCCGGGCCCGTATGGCTCGAAACCACA-3'	22
apMADSPFb2 5'-GAAACCAGAAATCCACCAAGAAACCAAT-3'	23
apMADSPFb3 5'-AGGGTATAGTGCTATTTCCACGTTTCATTC-3'	24
MADS2-5' 5'-CGCCGTCGCTGAACTCGATCC-3'	25
MADS2Nco 5'-CTCCCCATGGTCTCTCTAACA-3'	26
GenomeWalker AP1 primer: 5'-GTAATACGACTCACTATAGGGC-3'	27
GenomeWalker AP1 primer: 5'-ACTATAGGGCACGCGTGGT-3'	28

IT IS CLAIMED:

1. An isolated nucleic acid sequence comprising an apple fruit-associated promoter characterized by the ability to promote expression of a gene to which said apple fruit-associated promoter sequence is operably linked.

2. The apple fruit-associated promoter of claim 1 wherein said fruit-associated expression is ethylene regulated.

3. The apple fruit-associated promoter of claim 1 wherein expression is induced by changes in ethylene concentration in the fruit of a transformed plant, and said apple fruit-associated promoter or a functional portion thereof demonstrates greater activity during later stages of fruit development and/or early stages of fruit ripening.

4. The apple fruit-associated promoter of claim 1, wherein the apple fruit-associated promoter is selected from the group consisting of a *Thi-1* promoter comprising the nucleotide sequence presented as SEQ ID NO:1 or SEQ ID NO:2, a MADS2 promoter comprising the nucleotide sequence presented as SEQ ID NO:4 and a Fuji Thi 1.3-Actin fusion promoter comprising the nucleotide sequence presented as SEQ ID NO:3.

5. A plant expression vector comprising the promoter of claim 4.

6. The plant expression vector of claim 5, operably linked to a heterologous nucleic acid coding sequence.

7. The plant expression vector of Claim 6, operably linked to control sequences recognized by a host cell transformed with the vector.

8. A plant cell comprising the plant expression vector of claim 6 or 7.

9. A mature plant comprising the plant cell of claim 8.

10. A method of expressing a heterologous nucleic acid sequence in plant cells comprising:

- (a) transforming plant cells with a heterologous nucleic acid construct comprising an apple promoter of any one of claims 1 to 4 operably linked to a nucleic acid coding sequence;
- (b) culturing said plant cells in a culturing medium containing a selection agent to select for transformed plant cells;
- (c) in a manner effective to express the heterologous nucleic acid coding sequence; and
- (d) detecting expression.

11. A heterologous nucleic acid construct prepared by the process of operably linking:

- (i) an apple fruit-associated promoter of any one of claims 1 to 4 to;
- (ii) a transgene, wherein said promoter effects ethylene-regulated expression of said transgene.

5 12. The heterologous nucleic acid construct of claim 11, wherein the nucleotide sequence of said apple fruit-associated promoter is selected from the group consisting of a *Thi-1* promoter comprising the nucleotide sequence presented as SEQ ID NO:1 or SEQ ID NO:2, a MADS2 promoter comprising the nucleotide sequence presented as SEQ ID NO:4 and a Fuji Thi 1.3-Actin fusion promoter comprising the nucleotide sequence presented as SEQ ID NO:3.

10 13. A method for producing a transgenic plant, comprising;
introducing into progenitor cells of the plant the heterologous nucleic acid construct of claim 12
and growing the transformed progenitor cells to produce a transgenic plant.

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      10      20      30      40      50
AAGCTTTAGATCTCATGGGCGATGTGGGATGTCACAATCCACCCCCCTTA
      60      70      80      90     100
GGGGCCCGACGTCCCTCGTCATCACACTTCCGGCCAGGGATTGGCTCTAAT
      110     120     130     140     150
ACCATTTGTACATCCCGGCGGATCCACCACATCTCAAGCCCGTTCCA
      160     170     180     190     200
CCACCGTAGCATGATATTGTCCGCTTTGGGCTTACCATTCCCTCACGGTT
      210     220     230     240     250
TTGTTTTTGGGAACTCACGAGCAACTTCCTAGTGGGTACCCATCCTGGG
      260     270     280     290     300
AGTGTTTAACTTCGGAGTTCTTACGAAACCCGAAGCCAATGAGCTCCCAA
      310     320     330     340     350
AAGGTCTCGTGCTAAGTAGGGATGAGAATATACATTTAAGGATTACTCCC
      360     370     380     390     400
CTGGGCGATGTGGGATGTACAATTTGGGTAAGAAAATGACAAGATCAAAA
      410     420     430     440     450
TTAAAACTGTCAAATTTTATGCAAATTTGAAAAACAATTACAAAATCTTA
      460     470     480     490     500
AGGAAAGTATAACATTAGTGCTTTTTTTTTTGTTCGAAGAAGCATTAAACA
      510     520     530     540     550
TACAATTTGTATTGATATATTAATATGCAATGATTTTAAACATTAATGCA
      560     570     580     590     600
TTTTTTTTTCATTAATCCCTCCCTTCAAATATGCATAGAATTTAATGTAT
      610     620     630     640     650
ACATTAAAACTTTAATTAGGGGTGTTTTAGGCATCTAAAAAATGCAAAA
      660     670     680     690     700
TGTGTAAAGGCAAAATAGAATTAATGACTTTGCTTATGTGGAGCCTAGTCA
      710     720     730     740     750
TTAGGTTTTATTTAGATAAAAAAGACTATGTCAGGTTTTATGTAAAGAAAC
      760     770     780     790     800
TTGAGTTTCAAGAGCTAAAGTCATATTTTCAGTAGAAAATTAAACACATTA
      810     820     830     840     850
ATCAACACTTGAGTAATAAAATGATCATCAACAATCTAATCATTTGGTTT
      860     870     880     890     900
ACAAATTGAGAAATACTAAGGAGACTGTTTCAAAGTAAGACTTCCTATGA
      910     920     930     940     950
ACTCTCTATCACCTCATATTCTTGGCACAAAAATTTATAACATTAACATA
      960     970     980     990    1000
AGAAATTGTATCAACAACATAAAATGGCAGAAAGTTCGTAGAAAATCACAT
      >putative_TATA_box
      |
      1010     1020     1030     1040     1050
TCAAGATAATAGCCTTAGCAATTCCCTTATAAACTTTGTTCATCTAACATT
      1060     1070     1080     1090     1100
TCCCTCTCTATTCACTCTCCTCACACTCAAACACACACCGTGGACTGGTT
      1110     1120     1130     1140     1150
CATGCTTGCCACTTGTACCTCCCAAGAGGTTCTAGACCCTTCATATCCTA
      1160     1170     1180     1190     1200
TCCTCTTCCCACGTGTCCATCTTCAATTTTACATATACGTACCCCTCCTC
      1210     1220     1230     1240     1250
CTTAAATAACCACTCTCTTCACTTCCATCTTCTGACTTGCAAACGCTAAA
      1260     1270     1280     1290     1300
CCCCCAATCACCCCATCTTATCATCTTCTCTCTCTCCTCTCTCTCTCC
      1310     1320
TTCTCTCGCATCAATCCATGG
```

Fig. 1

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      10      20      30      40      50
AAGCTTTAGATCTCATGGGCGATGTGGGGATGTCACAATTTGGGTAAGAT
      60      70      80      90     100
AATGACAAGATCAAATTAAACTGTCAAATTTTAGGCAAATTTGAAAAAC
      110     120     130     140     150
AATTACAAAATCTTAAGGAAAGTATAACATTAGTGCTTTTTTTTGTTC
      160     170     180     190     200
AAGAAGCATTAACATACAATTTGTTATGATATATTAATATGCAGTGATTT
      210     220     230     240     250
TAAACATTAATGCATTTTTTTTTTCATTAACCCCTCCCTTCAAATATGCAT
      260     270     280     290     300
AGAATTTAATGTATACATTAAACTTTAATTAGGGGTGTTTtagGCATCT
      310     320     330     340     350
AAAAAATGCAAAATGTGTAAAGGCAAATAGAATTAATGACTTTGCTTAT
      360     370     380     390     400
GTGGAGCGTAGTCATTAGGTTTTATTTTAGATAAAAAGACTATGTCGGGTT
      410     420     430     440     450
TTATGTAAAGAACTTGAGTTTCAAGAGCTAAAGTCATATTTTCAGTAGA
      460     470     480     490     500
AATTAAACACATTAATCAACACTTGAGTAATAAAATGATCATCAACAATC
      510     520     530     540     550
TAATCATTTGGTTTACAAATTGAGAAATACTAAGGAGACTGTTTCAAAGT
      560     570     580     590     600
AAGACTTCCTATGAACCTCTCTATCACCTCATATTCTTGGCACAAAATTTT
      610     620     630     640     650
ATAACATTAACATAAGAATTGTATCAAAAACATAAAATGACAGAAAATTC
                                     putative_TATA_box
      660     670     680     690 |     700
GTAGAAAATCACATTCAAGATAATAGCCTTAGCAATTCCTTATAAACTT
      710     720     730     740     750
TGTCATCTAACATTTCCCTCTCTATTCACTCTCCTCACACTCAAACACAC
      760     770     780     790     800
ACCGTGGACTGGTTCATGCTTGCCACTTGTAACCTCCCAAGAGGTTCTAGA
      810     820     830     840     850
CCCTTCATATCCTATCCTCTTCCACGTGTCCATCTTCAATTTTACATAT
      860     870     880     890     900
ACGTCACCCCTCCTCCTTAAATAACCACTCTCTTCACTTCCATCTTCTGAC
      910     920     930     940     950
TTGCAAACGCTAAACCCCCAAATCACCCCATCTTATCATCTTCTCTCTCT
      960     970     980
CTCCCTCTCTCTCCTTCTCTCGCATCAATCCATGG

```

Fig. 2

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HindIII/BglII

AAGCTTTAGA TCTCATGGGC GATGTGGGAT GTCACAATCC ACCCCCCTTA
 GGGGCCCCGAC GTCCTCGTCA TCACACTTCC GGCCAGGGAT TGGCTCTAAT 100
 ACCATTTGTC ACATCCCGGC CCGGATCCAC CACATCTCAA GCCCGTTCCA
 CCACCGTAGC ATGATATTGT CCGCTTTGGG CTTACCATT C C C C C G G T T C C A
 TTGTTTTTGG GAACTCACGA GCAACTTCCT AGTGGGTCAC CCATCCTGGG 200
 AGTGTTTAAC TTCGGAGTTC CTACGAAACC CGAAGCCAAT GAGCTCCCAA 300
 AAGGTCTCGT GCTAAGTAGG GATGAGAATA TACATTTAAG GATTACTCCC
 CTGGGCGATG TGGGATGTCA CAATTTGGGT AAGAAAATGA CAAGATCAAA 400
 TTAAACTGT CAAGTTTTAT GCAAATTTGA AAAACAATTA CAAAATCTTA
 AGGAAAGTAT AACATTAGTG CTTTTTTTTT TGTTCCAAGA AGCATTAAACA 500
 TACAATTTGT TATGATATAT TAATATGCAA TGATTTTAAA CATTAAATGCA
 TTTTTTTTTC ATTAATCCCT CCCTTCAAAT ATGCATAGAA TTTAATGTAT 600
 ACATTAAAAC TTTAATTAGG GGTGTTTTAG GCATCTAAAA AAATGCAAAA
 TGTGTAAAGG CAAATAGAAT TAATGACTTT GCTTATGTGG AGCCTAGTCA 700
 TTAGGTTTTA TTTAGATAAA AAGACTATGT CAGGTTTTAT GTAAAGAAAC
 TTGAGTTTCA AGAGCTAAAG TCATATTTTC AGTAGAAATT AAACACATTA 800
 ATCAACACTT GAGTAATAAA ATGATCATCA ACAATCTAAT CATTTGGTTT
 ACAAAATTGAG AAATACTAAG GAGACTGTTT CAAAGTAAGA CTTCTATGA 900
 ACTCTCTATC ACCTCATATT CTTGGCACAA AATTTTATAA CATTAACATA
 AGAATTGTAT CAACAACATA AAATGGCAGA AAGTTCGTAG AAAATCACAT 1000
 TCAAGATAAT AGCCTTAGCA ATTCCCTTAT AAACCCCGT TTCTCTTCTT
 CCCTCTTCCT CTTATTCTCG TCTTTCAACT CACCTAGGTC GACAACACTC 1100
 ACTCCTCTCT CAGCCAGACC TTCTTCTTTG GAGGGTTGGC TCTTTCTTCT
 TCGTTTCGTT CTTCCCTTCT TCATTCATTC TCCTCTCTTT CATCCAAGGT 1200
 TTGTTTCTTC CTTCCCTTTT TTACCAAATC TTCTCACTTC CCTTACATTT
 TTCATCTGGG GTATCGTTCT TTTCCCAAAT TATGCTGCTT TCGTCTCTCA 1300
 TTTATCTACT TTATTGCTTT TAACTCATTT TCCCTTATGC GGTTCCTCAA
 TTTTGGCTGA TCTTGCTGTT TGTTTTGGA TTCTGTTTTA ATCGCCCTGG 1400
 ATCCGAGGTT TTTGTTCGTA CAATCTACCT AGATTCTTTC TGTTTGTGTTG
 CTGATCTGAA ATTTTCCATT TGGGTTTTGA TTGTCTGTGC TTACGGAAC 1500
 GAGATCTAGG ATTTGGAGTT GTGTACCTTT TTATTTCTGC ATGCAATTCT
 GTAATCCTGC ATAGCTGGAT GGCTTTCTGT TGATTAGTGC ATGCTTTGTT 1600
 TAGGACGAAC TGACTTGGAT TTTTCGTTGT CGATCTGTTC TATTTTTTGT
 TTTGCTGTTC TGGTTCATGC TTGGAATGAT TTAGTTGCTT TGTAATTTGT 1700
 AACTCTGCT TTTGTGTTAG TTCACGTAGC TTCTCGATCT GAAATTGGAT
 ATGGTTAGAG TTTATGGTCA GCTTGTGATC TTGCATTATG CAAAAATTGG 1800
 AACTTTAATC CTTTTCATTT GTAAGATCTT TAAGATATCT GATTACCTGG
 TTGATTTTTT TGTGTCTGGA TTATTTTATT TGTTTTGAAA GTAGTTTGTT 1900
 GGTTCCTCCT GTATTATTG CTGAATCGGG ATGATCAATT ATATGACGTG
 AATTTATGGA ATGTAAATGA ATGGTTTAAAG AGATTGCTTT GTGTGGCTTA 2000
 TTTATTCAAT TTCTATTTTT ACATCGTTTT GTGCAGGTTT TGAAAAAAA
GGGCCCATGG

Fig. 3

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      10      20      30      40      50
CGCCGTCGCTGAACTCGATCCGTGGCGCAGTCGAATGCCAGACCCAACTC
      60      70      80      90     100
AAAACCGAGTTTTTCCATTTTAAATTTTTTAAGTTTTTAATTATATAAAA
     110     120     130     140     150
ATATTTTTTTAATTATTACATACTTTTAGTTTCACATGGTAATGTTTAA
     160     170     180     190     200
TTAGATTTGTGGGACCCATTTATGTGTCACGTCAGCCCGTAACAGAATTT
     210     220     230     240     250
TTTACGAAATTATCACATTGATTTGCGACATCTATTTTCAGAGACTACAT
     260     270     280     290     300
TGATTGGTTTTTTAATTTTATAAACCATCTTAATGAAGTATGTCAATTTTA
     310     320     330     340     350
AAGATCATTTATTACAAAAACCCCTTTATTTAATTTTATATTGAAATACTA
     360     370     380     390     400
AAATATGATAAAATGTACTCGAATAGTTTAGTAGATAGGGTGGTGTATT
     410     420     430     440     450
TAGATACTTATTATTTTATTTTATACATACTCTTCTTAATTTCTAATCA
     460     470     480     490     500
GAAAATTGAATTAATAAAAAAATATCAATGAAAAATAATTTAACAAAAAT
     510     520     530     540     550
GTACAAAAATACAGAATGAACGTGGAATAGCACTATACCCTAGTAGATA
     560     570     580     590     600
TTGGATAAAATATATTATGGGTTTAAATTTGAAAAATATATGTGGTTTC
     610     620     630     640     650
GAGCCATACGGGCGCGGAATGACCGACTGTTGCAGTGCCTCTGGCCAAT
     660     670     680     690     700
CCCAACTCGACAACGTTTTTTGACGAAACCACTCTGGTTTTTCCAACCCAC
                                putative_TATA_box
      710      720      730 | 740      750
CCATTTCACTCTTACAGCGGTTTTTGAAATATCCTATAAATATATCATACA
     760     770     780     790     800
AATACAACAGAGAAATTTTTTTTTTTGTCAAAATATACAACAGAGAATTG
                                GAGA_box          GAGA_box
      810      820      830      840      850
AGTCACTCATATATAGACAGAGAAGGAGAGAGACCAGACCCCTACCTTAG
     860     870     880     890     900
AGAGAGAGAGAGAGCAGAAGCCATCTGTGTGTCAACTGGTTCTTTCTCTC
     910     920     930     940     950
CCATTTTCTTGGTTTCTTGGTGGGATTTCTGGTTTCTCTAAACTAAGAG
     960     970     980     990    1000
ATCAGTTCAGCAGGAACAACCGTATATATATTACTAGGATTATTAATTAT
    1010    1020    1030
TTATTTATAATAATAATAATTGTTAGAGAGACCATGG

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Fig. 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/11231

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/04; C12N 15/82

US CL : 536/23.1, 23.6, 24.1; 800/278

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 23.6, 24.1; 800/278

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EAST, EMBO Journals, STN, Genbank**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ATKINSON et al. Apple ACC-oxidase and polygalacturonase: ripening-specific gene expression and promoter analysis in transgenic tomato. Plant Molecular Biology. 1998, Vol. 38, pages 449-460, entire reference.	1-3
Y		10, 11

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Date of the actual completion of the international search

16 August 2000 (16.08.2000)

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28 AUG 2000

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